

REMARKS

Entry of the amendment is requested, whereby claims 38-58, 60, 61, and 63-66 will remain pending. Claims 38-41, 43-44, 58, and 60 are amended.

The amendments, inter alia, make changes directed to the Examiner's objections.

The remaining changes now require full hybridization and a specific, functional nucleotide sequence.

At page 6, line 23, application Serial Number 938 334 is incorporated by reference. This application is now U.S. Patent No. 5,405,940. For convenience, a copy of the front page of this patent is attached. This will show that the '940 patent describes MAGE-4 and MAGE-41 based tumor rejection antigens, and describes the sequence which encodes these. This sequence corresponds to those set forth by amendment in the claims. Hence, the amendment is supported by the specification, is proper, and should be entered.

The Examiner has rejected all claims as allegedly not satisfying the written description requirement. Similarly, the Examiner has rejected all claims under 35 U.S.C. § 112, first paragraph, as alleging that the claims are not enabled. Both rejections are traversed.

The Examiner argues that because Brasseur et al., *Int. J. Cancer*, 52:839-841 (1992) (which is not prior art), teaches that Northern Blotting did not suffice to distinguish various MAGE family members, PCR did. Therefore, the claims lack written description.

This rejection makes no sense. The art relied upon shows that while one method may have failed, another worked. Hence, the sequence can be distinguished, one from the other. Further, the claims now require a specific sequence NOT found in other MAGE molecules. Since PCR can distinguish the molecules, it is a matter of routine skill in the art to develop relevant, MAGE-4 and MAGE-41 specific primers, based upon this sequence.

With respect to the argument set forth at page 6, again this is not understood. The specification takes pains to explain how the invention differs from TSTA molecules, and hence this is irrelevant.

With respect to the argument presented over pages 7 and 8, the Examiner appears to be requiring structural characteristics. As was pointed out, supra, the claims require a sequence unique to the MAGE-4 family, and satisfy the alleged requirement.

Similarly, it is believed the enablement rejection cannot be maintained.

First of all, it has been shown that MAGE-4 and MAGE-41 molecules are processed to tumor rejection antigens, and the claims expressly recite such sequences. Other tumor rejection antigens are produced by MAGE-4 and MAGE-41 molecules, as is shown by the attached papers. While these are not prior art, if the Examiner is going to rely on non-prior art reference, so can applicants.

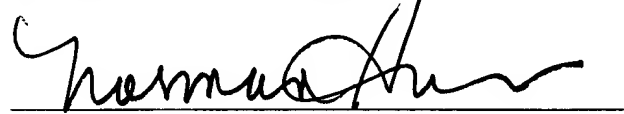
The arguments over pages 12-17 is believed to be irrelevant to the pending claims. None of this discussion deals with MAGE-4 or MAGE-41.

Further, since the claims expressly require the presence of a known and defined tumor rejection antigen, the references, to the extent they are prior art (and most of them are not), are irrelevant.

It is believed this application is in condition for allowance. If it is not, an interview is requested.

Respectfully submitted,

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A handwritten signature in black ink, appearing to read 'Norman D. Hanson', is written over a horizontal line.

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**Enclosures: Front Page of US Patent No. 5,405,940
Papers showing MAGE-4 and MAGE-41 Molecules**

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New MAGE-4 antigenic peptide recognized by cytolytic T lymphocytes on HLA-A1 tumor cells

Key words:

CTL; HLA-A1; *MAGE-4*; peptide; tetramer; tumor

Acknowledgments:

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Abstract: 'Cancer-germline' genes such as those of the *MAGE* family are expressed in many tumors and in male germline cells, but are silent in other normal tissues. They encode shared tumor-specific antigens, which have been used in small therapeutic vaccination trials of cancer patients. Gene *MAGE-4*, which is expressed in more than 50% of carcinomas of esophagus, head and neck, lung, and bladder, has two known alleles. Using PCR amplifications and digestions of the amplified product, we found that one third of the *MAGE-4*-positive samples expressed *MAGE-4a*. We folded HLA-A1 tetramers with peptide *MAGE-4a*_{109–177} EVDPASNTY, which is homologous to *MAGE-1* and *MAGE-3*-encoded peptides recognized on HLA-A1 by cytolytic T lymphocytes. Blood lymphocytes from an individual without cancer were directly labelled with these A1/*MAGE-4* tetramers. The very rare cells that were stained were sorted by flow cytometry and cloned. We isolated a cytolytic T-lymphocyte clone that lysed specifically cells pulsed with this *MAGE-4* peptide and HLA-A1 tumor cells expressing *MAGE-4a*, demonstrating that this antigenic peptide is processed efficiently in tumor cells. This peptide might therefore be useful for therapeutic antitumoral vaccination.

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The *MAGE* gene family comprises 24 functional genes divided into three clusters, named *MAGE-A*, *B*, and *C* (1–4). These genes are expressed in many human tumors of different histological types, but are silent in normal cells with the exception of male germline cells. Some of them are also expressed in placenta, including *MAGE-A4*, which will be referred to hereafter as *MAGE-4* (1). Male germline cells and placenta do not express MHC class I molecules and therefore cannot present antigens to cytolytic T lymphocytes (CTLs) (5). *MAGE*-encoded antigens are thus tumor-specific and of particular interest for cancer immunotherapy, as they are shared by many

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tumors. Clinical trials involving *MAGE* antigens have been performed in melanoma patients, and tumor regressions have been observed in a minority of patients (6–11).

There are two known alleles of gene *MAGE-4*: *MAGE-4a* and *MAGE-4b*. The putative proteins differ by a single amino acid (12). The distribution of the two alleles in the population is unknown. The gene contains eight alternative first exons that are spliced to unique second and third exons. The entire open reading frame is located in the third exon.

We reported previously the identification of two *MAGE-4* antigenic peptides recognized by CTLs: GVDGREHTV (*MAGE-4*_{230–239}) presented by HLA-A2 and SESLKMIF (*MAGE-4*_{156–163}) presented by HLA-B37 (13, 14). Both peptides are encoded by each of the two *MAGE-4* alleles. To produce these anti-*MAGE-4* CTLs, we had stimulated CD8 T cells from non-cancerous blood donors with autologous dendritic cells infected with adenoviruses or poxviruses, carrying the coding sequence of *MAGE-4*. Here, we have used a different strategy: a large number of blood lymphocytes were directly labelled with HLA-peptide tetramers folded with a *MAGE-4a*-encoded peptide. The very rare cells that were stained were sorted by flow cytometry, amplified in clonal conditions and analyzed further for tetramer labelling and specific lytic activity. We describe here the isolation of a CTL clone directed against a new *MAGE-4a* antigen.

Materials and methods

Cell lines, media, and reagents

The Epstein-Barr virus-transformed B (EBV-B) cell lines and the tumor cell lines were cultured in IMDM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Life Technologies). COS-7 cells were maintained in DMEM (Life Technologies) supplemented with 5% fetal calf serum. All the media were supplemented with 0.24 mM of L-asparagine, 0.55 mM of L-arginine, 1.5 mM of L-glutamine (AAG), 100 U/ml of penicillin and 100 µg/ml of streptomycin. Human recombinant IL-2 was purchased from Eurocetus (Amsterdam, the Netherlands), IL-7 from Peprotech (Rocky Hill, NJ), and GM-CSF (Leucomax) from Schering-Plough (Brinny, Ireland). Human recombinant IL-4, IL-6, and IL-12 were produced in our laboratory. One U/ml of IL-6 is the concentration needed to obtain half-maximal proliferation of mouse 7TD1 cells (15). Geneticin was purchased from Life Technologies. Cell line MZ2-MEL. 2.2-*MAGE-4* was obtained by cotransfecting into MZ2-MEL. 2.2, a pcDNA1/Amp plasmid (Invitrogen) that contained the coding sequence of *MAGE-4a* together with vector pSVtkneoβ, which contained the coding sequence conferring resistance to geneticin (14). CTL ROLE 521/A10, which is specific for

influenza basic polymerase 1 peptide VSDGGPNLY, was obtained after stimulation of blood cells with influenza basic polymerase 1 peptide VSDGGPNLY and sorting by flow cytometry after staining with influenza PB1.A1 tetramer.

Tetramer production and labelling with tetramers

Recombinant HLA-A1 molecules were folded *in vitro* with β2-microglobulin and peptide EVDPASNTY from *MAGE-4a*, or peptide VSDGGPNLY from the influenza basic polymerase 1. They were purified by gel filtration, biotinylated, and mixed as described (16) with Extravidin-PE (Sigma, St Louis, MT) for the HLA-A1/*MAGE-4a* tetramer, or streptavidin-APC (Molecular Probes, Eugene, OR) for the influenza control tetramer. For staining, cells were washed, resuspended at 20×10^6 cells per ml in PBS with 1% HS and incubated for 15 min at 4°C with HLA-A1 tetramers loaded with the *MAGE-4a* peptide (60 nM) or influenza peptide (20 nM). Anti-CD8 antibodies coupled to FITC (SK1 at 1/50, BD-Pharmingen, San Diego, CA), were added and after a further incubation for 15 min the cells were washed.

MACS and FACS sorting

Tetramer-labelled cells (10^7 cells/80 µl) were incubated with anti-PE microbeads (20 µl) according to the instructions of the manufacturer (Miltenyi Biotec, Bergish Gladbach, Germany), washed and sorted through a separation column inserted to a magnet in an AUTO-MACSTM at 0.5 ml/min (Miltenyi Biotec). FACS VantageTM cell sorter (BD-Pharmingen) was used for FACS sorting at 1 cell per microwell and FACSCaliburTM (BD-Pharmingen) for analysis of the T-cell clones, using CellquestTM software (BD-Pharmingen).

Culture conditions of cells sorted by flow cytometry

Sorted cells were cultured in U-bottomed microwells in 200 µl of IMDM supplemented with AAG, 10% human serum, IL-2 (100 U/ml), IL-4 (5 U/ml), and IL-7 (5 ng/ml). They were stimulated every 9–10 days with 1×10^3 irradiated (100 Gray) autologous peptide-pulsed dendritic cells, in the presence of IL-2 (100 U/ml), IL-4 (5 U/ml), IL-6 (1000 U/ml), IL-7 (5 ng/ml), IL-12 (10 ng/ml), phytohemagglutinin (PHA; 250 ng/ml; HA-16, Murex, UK) and irradiated allogeneic EBV-B cells (LG2-EBV) as feeder cells (3×10^4 cells/well). To prepare the stimulator cells, monocyte-derived immature dendritic cells were incubated overnight with 50 µg/ml of peptide EVDPANSTY in the presence of β2-microglobulin (2.5 µg/ml), IL-4 (200 U/ml), GM-CSF (70 ng/ml) and TNFα (1 ng/ml) and washed. After 3–5 weeks of proliferation, the CTL clones were re-stimulated every 1–2 weeks, in

2 ml of medium (5×10^5 CTL/well) with 2×10^5 irradiated LB34-MEL stimulator cells pulsed for 1 h with 1 μ g/ml of peptide in the presence of β 2-microglobulin, 1.5×10^6 feeder cells, IL-2, IL-4, and IL-7.

Dendritic cells

Peripheral blood was obtained from hemochromatosis patient LB672 as standard buffy coat preparations, which were laid down on a 15-ml of Lymphoprep layer (Axis-Shield PoCAS, Oslo, Norway) in 50-ml tubes. To minimize contamination of the PBMCs by platelets, the tubes were first centrifuged at 930 g for 20 min at room temperature. After removal of the top 20–25 ml, containing most of the platelets, the tubes were centrifuged at 430 g for 8 min at room temperature. The interphase containing the PBMCs was harvested and washed three times (or more) in cold phosphate buffer solution with 2 mM of EDTA in order to eliminate the remaining platelets. To generate autologous dendritic cells, PBMCs were left to adhere for 1 h at 37°C in culture flasks (FALCON, Becton Dickinson) at a density of 2×10^6 cells per cm^2 in RPMI 1640 supplemented with Hepes (2.38 g/l), AAG, antibiotics, and 10% FCS (hereafter referred to as complete RPMI medium). Nonadherent cells were discarded and adherent cells were cultured in the presence of IL-4 (200 U/ml) and GM-CSF (70 ng/ml) in complete RPMI medium. Cultures were fed on days 2 and 4 by removing one third of the volume and adding fresh medium with cytokines. They were frozen on day 5.

Transfection of COS cells and recognition assay based on TNF production

COS-7 cells (1.5×10^4) were distributed in flat-bottom microwells and cotransfected using 1 μ l of Lipofectamine (Life Technologies, Merelbeke, Belgium) with pcDNA1/Amp (50 ng) containing either a *MAGE-4a* cDNA or a HLA-A1 cDNA. Transfected cells were incubated for 24 h at 37°C and 8% CO_2 . The transfectants were then tested for their ability to stimulate the production of TNF by the CTL clone. Three thousand CTLs were added in the microwells containing either the COS-7 transfectants or tumor cells, in a total volume of 150 μ l of complete IMDM supplemented with 25–50 U/ml of IL-2. After 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on WEHI-164 clone 13 cells in a MTT colorimetric assay (17–19).

Expression of *MAGE-4a* and *MAGE-4b*

Total cellular RNA from tumor tissues and cell lines was extracted by the guanidine-isothiocyanate/cesium chloride procedure (20). Reverse transcription was performed on 2 μ g of total RNA in the presence of 200 units of M-MLV reverse transcriptase mixed with 4 μ l

of 5X First Strand Buffer (Life Technologies), 2 μ l of 20 mM oligo-(dT)₁₅, 20 units of RNasin (Promega, Madison, WI), 2 μ l of 100 mM of DTT (Life Technologies), 1 μ l of each dNTP at 10 mM of each (Takara, Shiga, Japan), and DEPC-treated water, in a total volume of 20 μ l. This mixture was incubated at 42°C for 90 min, then diluted to 100 μ l with water. PCR amplification was performed on 2.5 μ l (1/40th) of the reverse-transcribed RNA solution, in the presence of 0.625 units of Taq DNA polymerase in 1X PCR buffer (Takara), each primer at 0.4 μ M, and each dNTP at 100 μ M (Takara), in a total volume of 25 μ l. RNA integrity was checked by reverse transcription and amplification of the β -actin mRNA. Sense primer was 5'-GAG-CAGACAGGCCAACCG-3' (nucleotides –132 to –115 of the coding sequence of *MAGE-4a* and *MAGE-4b*) and antisense primer 5'-TCGCCCTCCATTGCAATTG-3' (nucleotides 629–643 of the coding sequence of *MAGE-4a* and *MAGE-4b*). Amplifications were performed as follows: after a first denaturation step at 94°C for 4 min, 30 cycles of amplification were performed (1 min at 94°C, 1 min at 65°C, 2 min at 72°C), then a final extension step terminated the reaction. The PCR products of 775 bp were analyzed by agarose gel electrophoresis directly and after digestion by TaqI (Fermentas, Vilnius, Lithuania; restriction site at nucleotides 374–378 of the coding sequence of *MAGE-4a* and *MAGE-4b*) or SmuI (Life Technologies; restriction site at nucleotides 513–523 of the coding sequence of *MAGE-4a*).

Cytotoxicity assay

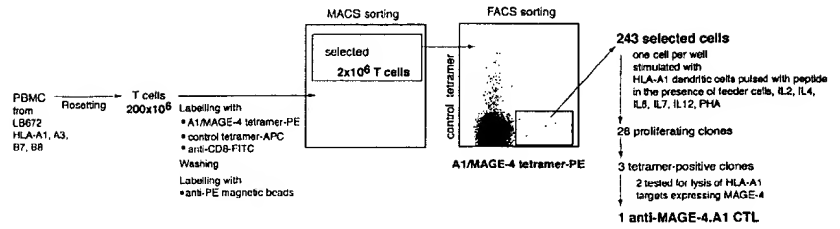
The EBV-B cells and tumor cells were labeled with 100 μ Ci of Na (⁶¹Cr)O for 1 h, washed and pulsed, if indicated, for 15 min with peptide. The CTLs were then added and chromium release was measured after incubation at 37°C for 4 h.

Results and discussion

MAGE-1 peptide EADPTGHSY and *MAGE-3* peptide EVDPIGHLY are presented to CTLs by HLA-A1. They are currently used in therapeutic vaccination trials (7, 21, 22). The homologous *MAGE-4a* peptide EVDPASNTY, which corresponds to the *MAGE-4a*_{169–177} protein sequence, contains potential anchor residues for HLA-A1, namely D in position 3 and Y at the carboxy-terminus (23, 24). The residue at position 5 of this peptide is the unique difference between the *MAGE-4a* and *MAGE-4b* putative proteins. *MAGE-4b* codes for peptide EVDPTSNTY.

HLA-A1 molecules produced in *E. coli* were folded with the *MAGE-4a* peptide, demonstrating that the peptide binds efficiently to HLA-A1. The HLA-peptide complexes were biotinylated, and

Fig. 1. Overview of the procedure to obtain anti-*MAGE-4A1* cytolytic T lymphocyte (CTL) clones. The control tetramer is a HLA-A1 tetramer containing an influenza peptide. MACS: magnetic cell sorting; FACS: fluorescence activated cell sorting. The cells represented on the FACS figure are CD8 T cells.



multimerized with avidin conjugated to phycoerythrin (PE). These multimers will be referred to as *A1/MAGE-4a* tetramers.

Isolation of CD8 T-cell clones labelled with tetramers

Two-hundred million T cells obtained from an individual without cancer were incubated with anti-CD8 antibodies, *A1/MAGE-4a* tetramers conjugated to PE, and control A1 tetramers folded with an influenza peptide and conjugated to allophycocyanin (APC) (Fig. 1). The cells were then incubated with an anti-PE antibody coupled to magnetic beads. Cells coated with beads were enriched by magnetic sorting. Approximately 2×10^6 cells were recovered. These cells were then introduced in a fluorescence-activated cell sorter, which selected the cells labelled with the *A1/MAGE-4a* tetramers conjugated to PE and cloned them at 1 cell per well. From the 243 microwells, 28 proliferating clones were obtained. Three of them were specifically labelled by *A1/MAGE-4a* tetramers. Only two clones, LB-709/D10 (clone D10) and LB-709/B1 (clone B1), proliferated enough to permit a functional analysis (Fig. 2).

Lysis of cells loaded with the *MAGE-4a* peptide

Clone D10, but not clone B1, had anti-*MAGE4a* lytic activity (Fig. 3). Titration of peptide EVDPASNTY revealed that half-maximal lysis of HLA-A1 target cells was obtained at a peptide concentration of 200 nM (Fig. 4). This is higher than for the previously identified *MAGE* antigenic peptides, for which values ranging from 0.05 to 100 nM were observed (22, 25–28). The *MAGE-4b* peptide,

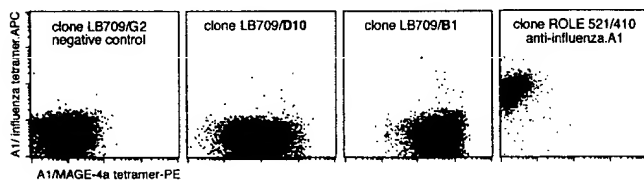


Fig. 2. Labelling of T-cell clones with *A1/MAGE-4a* tetramers. Cells were labelled for 15 min at 4°C with the *A1-MAGE-4a* tetramer (60 nM) conjugated to phycoerythrin (PE) and the control tetramer (20 nM) conjugated to allophycocyanin (APC). Anti-CD8 antibodies were then added for 15 min. The control HLA-A1 tetramer contains an influenza peptide, and specific staining with this tetramer was shown for anti-influenza A1 CTL clone ROLE 521/A10.

EVDPSTNTY, was not recognized by clone D10 (data not shown). The observation that lymphocytes, such as clone B1, can be labelled by a given peptide/MHC tetramer but fail to recognize peptide-pulsed cells have been described previously (29). This is probably an effect of the multimeric nature of the tetramers, which permits stable binding despite low affinity.

Recognition of cells expressing *MAGE-4a*

COS-7 cells cotransfected with a *MAGE-4* and *HLA-A1* cDNA constructs stimulated clone D10 to produce TNF, indicating that the *MAGE-4* antigen could be processed in these cells (Fig. 5). To identify the tumor cells expressing *MAGE-4a*, a fragment of 775 nucleotides that includes the peptide-coding sequence was amplified by PCR and digested by restriction enzymes *TaqI* and *SmaI* (Fig. 6). Only the *MAGE-4a* products can be digested by *SmaI* whereas *TaqI* can digest PCR products obtained from the two alleles. Sixteen out of

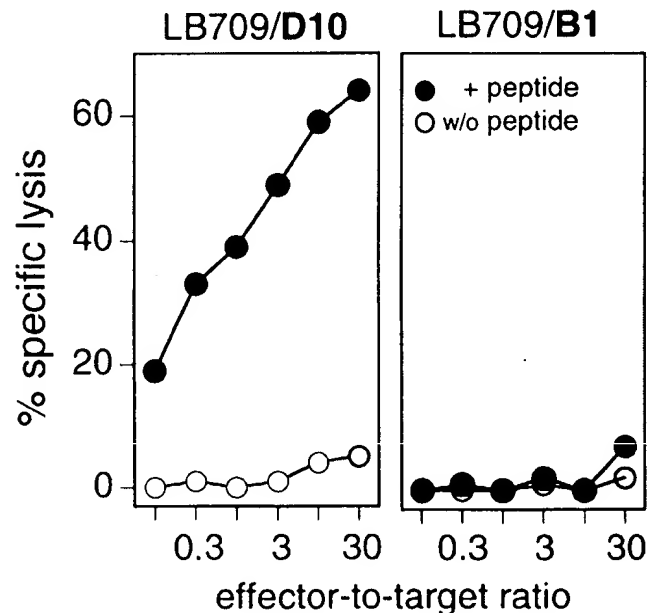


Fig. 3. Lysis of HLA-A1 targets loaded with *MAGE-4a* peptide EVDPASNTY. HLA-A1 BM21-Epstein-Barr virus-transformed B cells were ^{51}Cr -labelled for 1 h, incubated with 1 µg/ml of peptide for 15 min and incubated with the cytolytic T lymphocytes (CTLs) at indicated effector-to-target ratios. Chromium release was measured after 4 h.

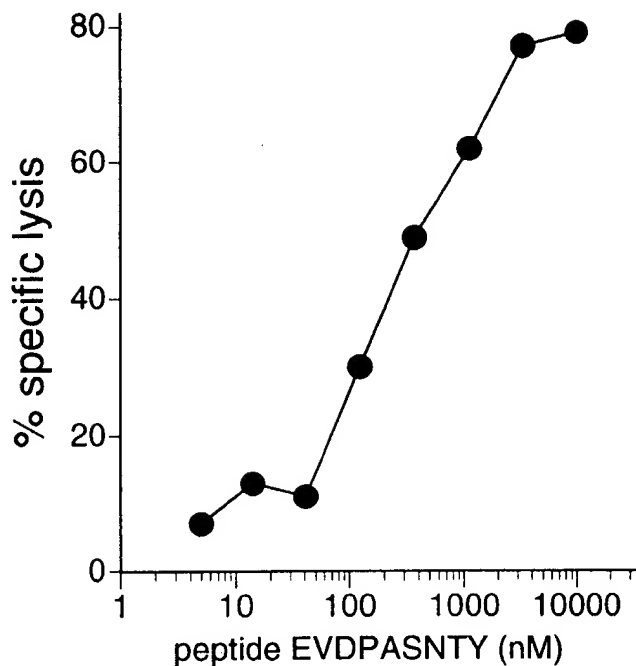


Fig. 4. Titration of the *MAGE-4a* peptide. BM21-Epstein-Barr virus-transformed B cells were ^{51}Cr -labelled and incubated for 15 min with threefold dilutions of the synthetic peptide. Cytolytic T lymphocyte D10 was subsequently added at an effector-to-target ratio of 5:1. Chromium release was measured 4 h later. The concentrations indicated in the figure correspond to the concentrations during the 4-h incubation.

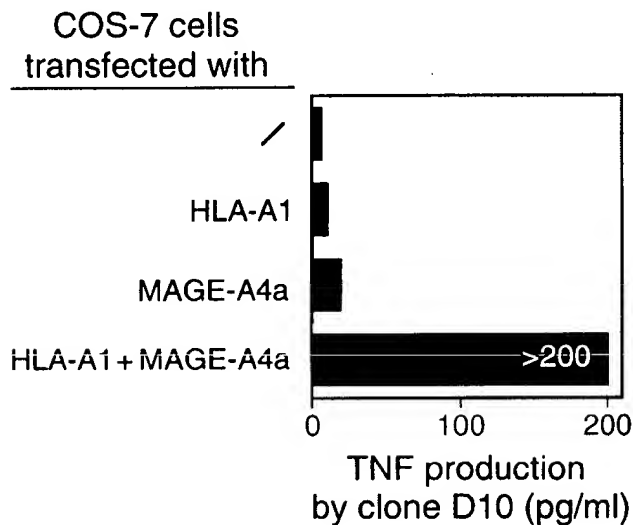


Fig. 5. Recognition of cells transfected with *MAGE-4a*. COS-7 cells were transiently transfected with a *MAGE-4a* and a HLA-A1 coding sequence, both inserted in expression vector pcDNA1/Amp. Transfections were performed with 15,000 COS-7 cells, 50 ng of each cDNA and 1 μl of Lipofectamine. One day after transfection, 3000 D10 cytolytic T lymphocytes were added to the transfected cells. TNF production was measured after overnight coculture by testing the toxicity of the supernatants for TNF-sensitive WEHI 164 clone 13 cells.

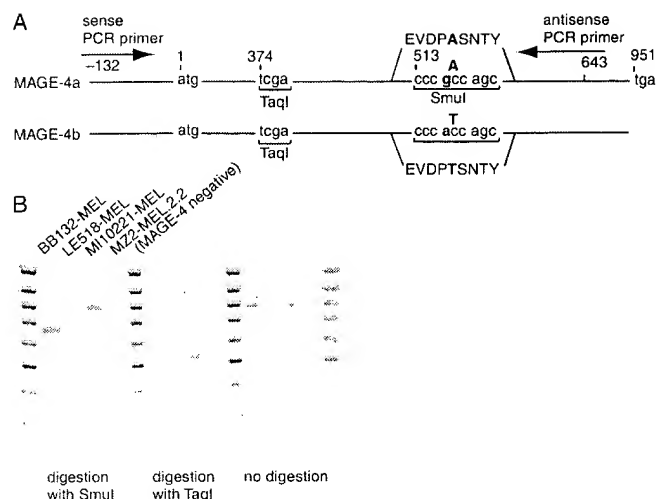


Fig. 6. One-restriction enzyme can distinguish *MAGE-4a* from *MAGE-4b*. (A) Nucleotides are numbered starting from the start codon. Restriction enzyme Smu 1, also named Fau 1, digests only *MAGE-4a* products, whereas restriction enzyme Taq 1 digests both *MAGE-4a* and *MAGE-4b* products. (B) Digestion of the PCR products. RNA was extracted from *MAGE-4*-expressing cell lines BB132-MEL, LE518A1-MEL and MI0221-MEL, which were used as targets in Fig. 7. RNA was also extracted from cell line MZ2-MEL.2.2, which do not express *MAGE-4*. RT-PCR was performed according to the protocol described in (A). PCR products were analyzed by agarose gel electrophoresis after digestion by Smu 1, and Taq 1, or without digestion.

the 46 tumor samples (35%) that were previously found to be positive for the expression of *MAGE-4* were found to express *MAGE-4a*. Eleven out of 37 *MAGE-4*-positive tumor cell lines (30%) expressed the *MAGE-4a* allele. Two of these tumor cell lines were derived from

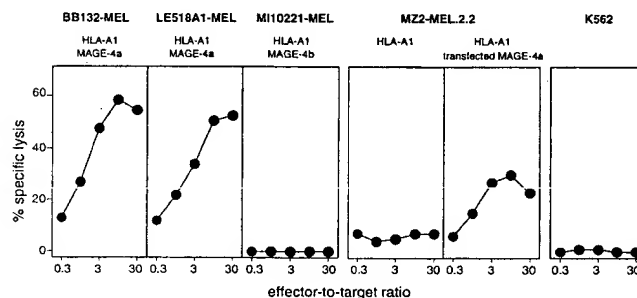


Fig. 7. Lysis of HLA-A1 tumor cell lines expressing *MAGE-4a*. Melanoma cell lines LE518A1 and BB132 express the *MAGE-4a* and HLA-A1 genes. Melanoma cell line MI10221 expresses the *MAGE-4b* and HLA-A1 genes. The MZ2-MEL.2.2.MAGE-4 cell line was obtained by stable transfection of the *MAGE-4a* coding sequence of HLA-A1 melanoma cell line MZ2-MEL.2.2. Cell line K562 is a target for natural killer cells. Target cells were ^{51}Cr -labelled and, if indicated, pulsed for 5 min with 1 $\mu\text{g}/\text{ml}$ of peptide EVDPASNTY. They were then incubated for 4 h with cytolytic T lymphocyte clone D10 at the indicated effector-to-target ratios. Chromium release was measured after 4 h.

a HLA-A1 patient. They were lysed by clone D10 whereas a HLA-A1 melanoma cell line expressing *MAGE-4b* was not lysed (Fig. 7). A HLA-A1 tumor cell line, MZ2-MEL.2.2, transfected with a *MAGE-4a* coding sequence was also lysed.

The new *MAGE-4a* antigenic peptide described here is presented by HLA-A1 molecules, which are expressed in the different major ethnic groups: Black (9%), Caucasoid (26%), Oriental (7%), and Amerindian (11%) (30). The identification of a large number of antigenic peptides presented by HLA class I and class II is likely to be important for the future

of clinical trials with defined antigenic peptides. A large set of peptide/HLA combinations will widen the set of eligible patients. It will also facilitate the design of concurrent immunizations against several antigens. Such immunizations could increase the primary antitumor efficacy of the vaccine and decrease the risk of tumor escape by loss of antigen expression. Identification of additional *MAGE-4* antigenic peptides will be important because a number of tumors express *MAGE-4* without expressing *MAGE-A1* and *MAGE-A3*. This is the case for carcinomas of the lung (15%), head and neck (14%), esophagus (11%), and bladder (10%).

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A MAGE-A4 peptide presented by HLA-A2 is recognized by cytolytic T lymphocytes

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The *MAGE*-encoded antigens that are recognized by cytolytic T lymphocytes (CTL) are shared by many tumors and are strictly tumor specific. Clinical trials involving therapeutic vaccination of cancer patients with *MAGE* antigenic peptides or proteins are in progress. To increase the range of patients eligible for therapy with peptides, it is important to identify additional *MAGE* epitopes. We have used a method to identify CTL epitopes, which selects naturally processed peptides. CD8⁺ T cells, obtained from individuals without cancer, were stimulated with autologous dendritic cells infected with a recombinant adenovirus containing the *MAGE-A4* coding sequence. Responder cell microcultures that specifically lysed autologous EBV-transformed B cells infected with vaccinia-*MAGE-A4* were cloned using autologous stimulator cells infected with a *Yersinia enterocolitica* carrying the *MAGE-A4* sequence. An anti-*MAGE-A4* CTL clone was obtained and the epitope was found to be decapeptide GVDGREHTV (amino acids 230–239) presented by HLA-A2 molecules. The CTL clone lysed HLA-A2 tumor cells expressing *MAGE-A4*. This is the first reported antigenic peptide encoded by *MAGE-A4*. It may be valuable for cancer immunotherapy because *MAGE-A4* is expressed in 51 % of lung carcinomas and 63 % of esophageal carcinomas, whereas about 50 % of Caucasians and Asians express HLA-A2.

Key words: MAGE / Adenovirus / Cytotoxic T lymphocyte / Tumor / Antigen

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1 Introduction

CTL directed against human tumor cells have been obtained by cultivating irradiated tumor cells with autologous lymphocytes from cancer patients [1, 2]. Several of these CTL have been used as tools to isolate genes that code for the antigens recognized on the tumors, such as those of the *MAGE* gene family, which includes at least 17 related genes, namely *MAGE-1* to *12* (now named *MAGE-A1* to *A12*), *MAGE-B1* to *B4* and *MAGE-C1* [3–7]. Genes of this family are expressed in tumors of various histological types but not in normal tissues, except male germ-line cells, which do not present HLA class I molecules and are therefore unable to present antigens to CTL [8, 9]. Hence, antigens encoded by *MAGE* genes should be strictly tumor specific.

[1 19756]

Abbreviations: DC: Dendritic cells **IMDM:** Iscove's modified Dulbecco's medium **EBV-B:** Epstein-Barr virus-infected B cells **ALVAC-MAGE-A4:** Avian poxvirus ALVAC containing the *MAGE-A4* coding sequence **MOI:** Multiplicity of infection

The gene *MAGE-A4* presents two alleles, *MAGE-A4a* and *MAGE-A4b*, which code for proteins differing by a single amino acid [4]. It contains eight alternative first exons that are spliced to unique second and third exons. The entire open reading frame is always located in the third exon [10]. The role of these different first exons, each with their own promoter, is unknown. *MAGE-A4* is not only expressed in male germ-line cells but also in the placenta, which does not present HLA class I molecules [11]. In addition, the placenta produces a tryptophan-catabolizing enzyme, which suppresses T cell activity [12]. *MAGE-A4* is frequently expressed in carcinomas of the esophagus (63 %), the head and neck (53 %), the lung (51 %) and the bladder (33 %), and also in metastatic melanomas (28 %). When a tumor sample is found to be positive for *MAGE-A4*, this gene is usually expressed at a very high level. *MAGE-A4*-derived antigens may thus be valuable for cancer immunotherapy but no such antigen has been identified so far.

Several strategies can be considered to find CTL epitopes encoded by *MAGE-A4*. A first approach requires the location of candidate peptides carrying consensus

anchor motives for a certain HLA in the sequence of the MAGE-A4 protein. These peptides are then tested for HLA binding and the best binders are loaded on APC to stimulate T lymphocytes *in vitro* [13, 14]. Epitopes encoded by MAGE-A1, A2, A3, and B2 have been identified by this approach [14–17]. Although straightforward, the major drawback of this approach is that many peptide-specific CTL do not recognize HLA-matched tumor cells expressing the protein endogenously [18, 19]. We followed a second approach that ought to lead to epitopes that are well processed. Here, dendritic cells (DC) were modified to express MAGE-A4 and used to stimulate autologous CD8⁺ T lymphocytes *in vitro*. DC are potent APC and they can activate naive T lymphocytes [20]. There is so far no effective method to transfect human DC. However, viral delivery vectors have been used successfully: it has been shown that DC can be infected with recombinant adenoviruses and poxviruses, and that infection with a recombinant adenovirus leads to a high expression of the transgene, which remains elevated for more than 1 week [21, 22].

We report here the identification of a MAGE-A4 antigenic peptide presented to CTL by HLA-A2 molecules, using an approach based on DC infected with an adenoviral vector encoding MAGE-A4.

2 Results

2.1 Activation of a CTL specific for a MAGE-A4 epitope

The experiments were performed with blood cells from an individual without cancer, hemochromatosis patient LB1137. DC were obtained by culturing blood monocytes for 4–6 days in medium supplemented with GM-CSF and IL-4. Cells prepared by this procedure have been referred to as "immature DC" [23, 24]. DC were infected with an adenovirus engineered to contain the MAGE-A4 sequence under the influence of a CMV promoter (adeno-MAGE-A4). Infected DC were distributed in microwells to stimulate autologous CD8⁺ T cells, once per week for 3 weeks. Since one of the essential features of a mature DC is to produce IL-12, the medium was supplemented with IL-12. Recently, we have also used mature stimulator DC, obtained by treating immature DC for 2 days with TNF α , IL-1 β , IL-6, and PGE₂ [25]. Very high proliferation of T cells was observed in all the microcultures, but we did not obtain effectors with anti-MAGE lytic activity. We have tentatively concluded that the anti-MAGE CTL were overwhelmed by other T cells that proliferated very rapidly after stimulation by mature DC and, therefore, we continue to use immature stimulator DC in medium supplemented with IL-12.

Responder cells of each microculture were tested on day 21 for their lytic activity on autologous EBV-transformed B (EBV-B) cells infected with a vaccinia virus encoding MAGE-A4 (vaccinia-MAGE-A4). The reason for using vaccinia-MAGE-A4 instead of adeno-MAGE-A4 was primarily that adenoviral vectors rarely infect EBV-B cells. Moreover, vaccinia-infected targets are not lysed by CTL that would have been activated by antigens derived from the adenoviral vector.

A microculture displaying anti-MAGE-A4 reactivity was cloned by limiting dilution in the presence of autologous EBV-B stimulator cells, IL-2 and allogeneic EBV-B feeder cells. EBV-B stimulators were used for the cloning step because of the limited supply of autologous DC. To stimulate only the anti-MAGE-A4 CTL clones, while avoiding the proliferation of CTL against the adenoviral vector, we infected the irradiated EBV-B stimulators with recombinant *Yersinia enterocolitica* bacteria producing MAGE-A4 (Yersinia-MAGE-A4). These recombinant bacteria expressed a YopE-MAGE-A4 fusion gene allowing the translocation of a MAGE-A4 fusion protein into the cytosol of mammalian cells (see Sect. 4.3). We had previously verified that HLA-A1 EBV-B cells infected with Yersinia-MAGE-A1 were capable of stimulating the release of IFN- γ by CTL clone 82/30 [3], which is directed against a MAGE-A1 epitope presented by HLA-A1 (data not shown). After a few weekly restimulations, the growing CTL clones were tested for their lytic activity. Several clones were obtained which lysed autologous EBV-B cells only when infected with vaccinia-MAGE-A4. Results are shown for representative CTL clone H4/13, hereafter referred to as CTL clone 13 (Fig. 1).

We compared DC infected with adeno-MAGE-A4 or with a canarypox virus containing the coding sequence of

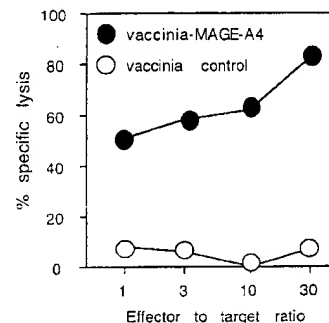


Figure 1. Lysis by CTL clone 13 of autologous EBV-B cells infected with vaccinia-MAGE-A4. EBV-B cells were infected for 2 h with the vaccinia vector, ⁵¹Cr-labeled for 1 h and incubated with the CTL at the indicated E/T ratios. Chromium release was measured after 4 h.

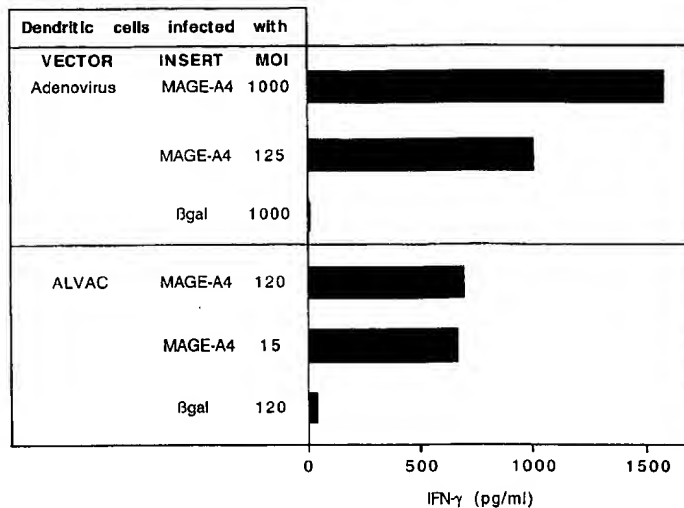


Figure 2. Recognition of DC infected with the viral constructs expressing *MAGE-A4*. HLA-A2 DC were infected with adeno-*MAGE-A4*, ALVAC-*MAGE-A4*, or control vectors containing the β -galactosidase (β gal) reporter gene, at the indicated MOI. When DC were infected with an adenoviral vector at a MOI of 1 000, the level of infection was usually higher than 80 % as assessed with a β -galactosidase reporter. The plateau of infection was obtained for ALVAC at a MOI of 15, which usually gives 30–40 % infected cells. Twenty-four hours after infection, 2 500 CTL 13 were added and the concentration of IFN- γ in the supernatant was measured after 24 h.

MAGE-A4 (ALVAC-*MAGE-A4*) for their ability to stimulate CTL clone 13. DC infected with adeno-*MAGE-A4* stimulated the secretion of a high amount of IFN- γ by CTL 13, whereas cells infected with ALVAC-*MAGE-A4* were less efficient stimulators (Fig. 2). In a few other experiments, DC infected with ALVAC-*MAGE-A4* were used to stimulate anti-*MAGE-A4* CTL precursors and microcultures displaying anti-*MAGE-A4* reactivity were obtained (data not shown).

2.2 A *MAGE-A4* peptide presented by HLA-A2

Patient LB1137 was typed HLA-A2, A3, B4402, B60, Cw3, Cw5. The recognition of autologous EBV-B cells infected with vaccinia-*MAGE-A4* by CTL clone 13 was inhibited in the presence of anti-HLA-A,B,C and anti-HLA-A2 mAb, demonstrating that the *MAGE-A4* epitope was presented by HLA-A2 molecules (data not shown). To confirm that the epitope recognized by this CTL was encoded by *MAGE-A4*, COS-7 cells were cotransfected with the *MAGE-A4* cDNA and the HLA-A2 gene. These transfectants stimulated CTL clone 13 to produce TNF (Fig. 3).

To identify the region of *MAGE-A4* that codes for the peptide recognized by clone 13, we constructed truncated coding sequences of *MAGE-A4* of various lengths.

PCR reactions were performed using a *MAGE-A4* cDNA as the template, an upstream primer consisting of the first nucleotides of the open reading frame of *MAGE-A4*, and eight downstream primers. This led to the amplification of eight fragments of *MAGE-A4*, the longer one con-

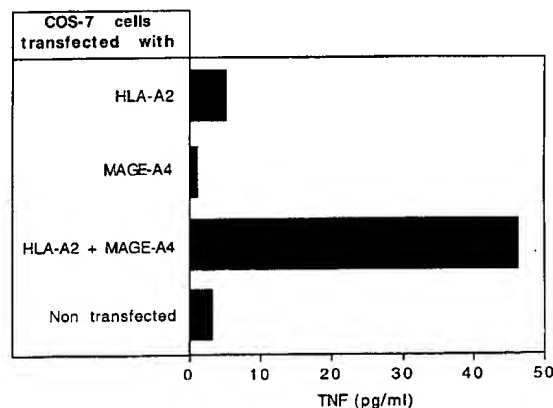


Figure 3. Recognition by CTL clone 13 of a *MAGE-A4* antigen presented by HLA-A2. COS-7 cells were transfected with expression vectors containing the HLA-A2 gene and the *MAGE-A4* cDNA. CTL 13 cells (2000) were added to the transfectants and the TNF content of the supernatant was estimated after 24 h by testing its toxicity on WEHI-164 clone 13 cells.

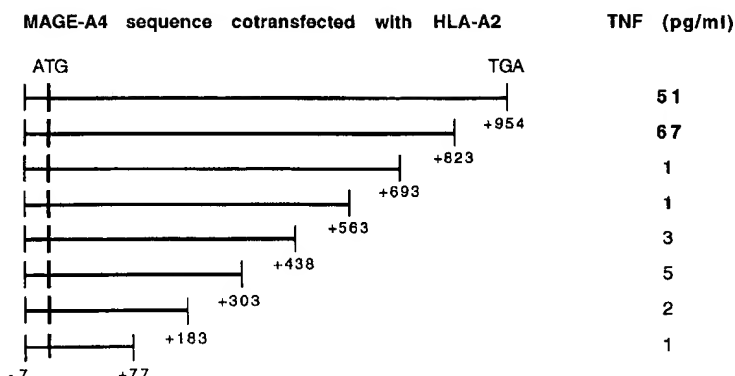


Figure 4. Identification of a *MAGE-A4* fragment coding for the antigen recognized by CTL clone 13. PCR reactions were performed on the *MAGE-A4* cDNA to obtain eight truncated *MAGE-A4* cDNA that were cloned in an expression vector. Nucleotides were numbered, starting from the first nucleotide of the open reading frame. HeLa cells (20 000) were cotransfected with expression vectors containing the HLA-A2 gene and the *MAGE-A4* fragments. CTL 13 cells (2000) were added 24 h later to the transfectants and the TNF concentration of the supernatant was measured 20 h later, by testing its toxicity on WEHI-164 clone 13 cells.

taining the entire open reading frame of *MAGE-A4*. These fragments were inserted into an expression vector and transfected into HeLa cells, along with the HLA-A2 gene, to test their ability to code for the antigen. Transfection with the two longer inserts stimulated the secretion of TNF by CTL 13, while the other constructs were negative (Fig. 4). This indicated that the C terminus of the peptide had to be encoded by nucleotides 693 to 823 of the open reading frame. The sequence of the *MAGE-A4* protein encoded by this region was screened for the presence of a peptide carrying consensus anchor residues for HLA-A2 (L or V in position 2 and I, L, V, or M in position 9 or 10) [26]. We found only one peptide of ten amino acids, GVDGREHTV (amino acids 230–239), that satisfied this criterion. Peptide GVDGREHTV was found to sensitize autologous target cells to lysis by clone 13, while nonapeptide VVDGREHTV did not. Half-maximal lysis was obtained at a peptide concentration of 30 nM (Fig. 5). This result is in the same range as for other *MAGE* peptides: 5 nM for the *MAGE-A1* peptide presented by HLA-A1, and 25 nM for the *MAGE-A1* peptide presented by HLA-Cw*1601 [27, 28].

2.3 Recognition of HLA-A2 tumor cell lines expressing *MAGE-A4*

Since we used DC expressing *MAGE-A4* to activate CTL clone 13, it was important to verify that tumor cells also efficiently processed the *MAGE-A4* antigen. Three HLA-A2 melanoma and a sarcoma cell line expressing *MAGE-A4* were tested and all were found to be lysed by CTL 13 (Fig. 6).

3 Discussion

The procedure described here appears to be efficient for the activation of anti-*MAGE* CTL precursors and does not require blood cells from cancer patients. We have observed better survival and stimulation capacities for DC infected with an adenoviral vector as compared with poxvirus-infected DC. However, we cannot conclude from our few experiments that adenovirus-infected DC are better stimulators for activation of anti-*MAGE* CTL precursors from individuals without cancer. A potential

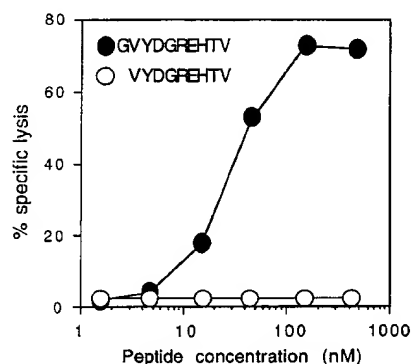


Figure 5. Lysis by CTL clone 13 of autologous EBV-B cells incubated with peptide GVDGREHTV. EBV-B cells were ^{51}Cr -labeled for 1 h and incubated with CTL 13 at an E/T ratio of 10:1 in the presence of the *MAGE-A4* peptide at the concentrations indicated. Chromium release was measured after 4 h.

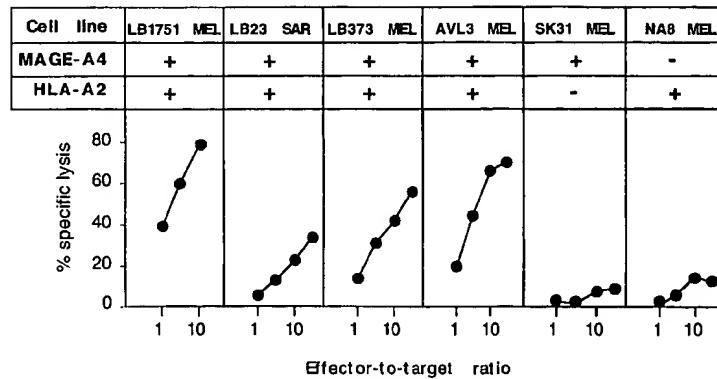


Figure 6. Lysis by CTL clone 13 of HLA-A2 tumor cell lines expressing MAGE-A4. Target cells were ^{51}Cr -labeled for 1 h and incubated with CTL at various E/T ratios. Chromium release was measured after 4 h. MEL stands for melanoma and SAR for sarcoma.

drawback of our approach, which is based on the use of a recombinant virus for antigen delivery, is that CTL precursors directed against viral epitopes can easily be activated. We attempted to circumvent this problem by using different vectors for the stimulation of the microcultures, for testing the lytic activity with the responder T cells, and for the cloning step.

Interestingly, the MAGE-A4-derived peptide, GVDG-REHTV, and the recently identified MAGE-A10 peptide, GLYDGMEHL, which is also presented by HLA-A2 but not recognized by CTL 13, are located in homologous regions of the protein [29]. This was also observed for the MAGE-A3 and MAGE-A1 antigens presented by HLA-A1 [27, 30]. This may suggest that some fragments are processed more efficiently than others. The identification of a series of additional antigens in these MAGE proteins may help to define rules for the processing of proteins into antigenic peptides.

After infection with viral vectors containing an entire *MAGE* coding sequence, only those antigenic peptides processed efficiently by the infected DC are able to activate anti-MAGE CTL. These peptides are most likely those that are also well processed and presented at the surface of MAGE⁺ tumor cells. Accordingly, CTL clone 13 lysed all four HLA-A2 tumor cell lines expressing *MAGE-A4*. The MAGE-A4 epitope described here is also efficiently processed by DC and EBV-B cells infected with a viral vector coding for *MAGE-A4*. In contrast, the MAGE-A3_{271–279} epitope presented by HLA-A2, which was identified by the peptide approach [15], appeared to be poorly processed: EBV-B cells infected with vaccinia-MAGE-A3 were not lysed by CTL clone 297/22 (data not shown). This is probably due to the inaccurate cleavage of the MAGE-A3 protein during its processing [31]. Even

though some tumors that appeared to express a high level of *MAGE-A3* showed significant lysis by CTL clone 297/22 and by other CTL clones directed against the same peptide [15, 32, 33], we feel that the MAGE-A4 epitope presented here could be a better candidate for immunotherapy, because it is processed more efficiently.

MAGE-A4 is expressed in a significant proportion of tumors, and 49 % of Caucasians and Asians express the HLA-A2 allele. This MAGE-A4 epitope could be particularly interesting for those patients whose tumor expresses *MAGE-A4* but not *MAGE-A1* or *MAGE-A3*, which is the case in a number of carcinomas of the lung (15 %), the head and neck (14 %), the esophagus (11 %), and the bladder (10 %). Different modes of immunization can be envisaged: a synthetic peptide, DC pulsed with this peptide, a MAGE-A4 protein, or viral vectors encoding the epitope. In clinical trials, the availability of monoclonal and polyclonal anti-MAGE-A4 antibodies may facilitate the detection of the MAGE-A4 protein, both in tissue sections and in the serum of cancer patients [34, 35].

In future trials, it will be essential to have reliable monitoring of the CTL response against the immunizing antigen. One possibility, which unfortunately narrows the analysis to certain epitopes, is the use of relevant peptides to label T cell receptors directly with soluble HLA tetramers presenting the relevant peptide [36]. HLA-A2 tetramers folded with melanoma peptides have been shown to detect anti-Melan-A and anti-tyrosinase CTL in melanoma-infiltrated lymph nodes [37]. This approach requires prior identification of these antigenic peptides. An HLA-A2 tetramer folded with this MAGE-A4 peptide can now be constructed, tested with our CTL and then used for monitoring.

4 Materials and methods

4.1 Cell lines and peptides

Melanoma cell lines LB1751-MEL, AVL3-MEL, LB373-MEL.4.0, LB23-SAR clone 2, SK31-MEL and NA8-MEL were grown in Iscove's modified Dulbecco's medium (IMDM) containing 10 % FCS and supplemented with 0.24 mM L-asparagine, 0.55 mM L-arginine and 1.5 mM L-glutamine (AAG); for LB1751-MEL and AVL3-MEL the medium was also supplemented with Hites [38]. COS-7 cells and HeLa cells were maintained in DMEM with 10 % FCS. WEHI-164 clone 13 cells were cultured in RPMI with 5 % FCS. Culture media were purchased from Gibco-BRL (Merelbeke, Belgium). Peptide were synthesized on solid phase using Fmoc for transient N-terminal protection and were characterized using mass spectrometry. All peptides were > 80 % pure, as indicated by analytical HPLC. Lyophilized peptides were dissolved in DMSO and stored at -20°C .

4.2 Poxviruses and adenoviruses

Recombinant canarypoxvirus ALVAC-MAGE-A4 (vCP1544) and ALVAC- β gal (vCP325) and the vaccinia viruses encoding MAGE-A4 (vP1545) were produced by Virogenetics (Troy, NY). The plasmid pAd-CMVlcpA-MAGE-A4 (containing the MAGE-A4 cDNA under the control of the CMV promoter) was obtained by inserting the complete MAGE-A4 cDNA into the NotI site of vector pAd-CMVlcpA (kindly provided by Celia Garcia-Martinez and Thierry Ragot, Institut Gustave Roussy, France). The recombinant adenovirus adeno-MAGE-A4 was constructed by *in vivo* homologous recombination in cell line 293 between pAd-CMVlcpA-MAGE-A4 and adeno- β gal genomic DNA [39]. The recombinant adenovirus was plaque purified, propagated in cell line 293 and purified by double cesium chloride density centrifugation.

4.3 Construction of a recombinant *Yersinia* and infection of EBV-B cell lines

The wild-type *Y. enterocolitica* is an extracellular bacterium causing gastro-intestinal syndromes in humans. *Y. enterocolitica* adheres to the surface of target cells and possesses a virulence apparatus, called the Yop virulon, which enables the translocation of toxic effector proteins, including YopE, into the cytosol of the host cell [40, 41]. A new strain, MRS40 (pABL403), has recently been constructed, in which the genes encoding toxic Yop proteins are mutated or truncated [42]. Interestingly, this polymutant strain maintains its ability to translocate proteins in fusion with a truncated YopE into the cytosol of eukaryotic cells, but does not elicit cytotoxicity and can therefore be used as a vector to inject a protein into the cytosol of eukaryotic cells. The sequence encoding protein MAGE-A4 was inserted in frame with a sequence encoding the first 130 amino acids of YopE, which was

inserted into vector pMS621 [40]. Plasmid pMS621-MAGE-A4 was electroporated in *Escherichia coli* strain SM10 and was mobilized by SM10 into *Y. enterocolitica* MRS40 (pABL403). Recombinant MRS40 (pABL403) clones were selected on agar-containing medium, supplemented with 35 $\mu\text{g/ml}$ nalidixic acid, 1 mM sodium m-arsenite and 12 $\mu\text{g/ml}$ chloramphenicol [43].

One colony of *Y. enterocolitica* MRS40 (pABL403) containing pMS621-MAGE-A4 was grown overnight at 28°C in LB medium supplemented with 35 $\mu\text{g/ml}$ nalidixic acid, 1 mM sodium m-arsenite and 12 $\mu\text{g/ml}$ chloramphenicol. This culture was diluted in order to obtain an OD of 0.2 at 600 nm and cultured at 28°C for approximately 2 h. The bacteria were then washed in 0.9 % NaCl and resuspended at 10^8 per ml in 0.9 % NaCl, assuming that a culture giving an OD₆₀₀ equal to 1 contains 5×10^8 bacteria per ml. Irradiated EBV-B cells were resuspended at 10^6 in 3.8 ml of RPMI without antibiotics, supplemented with 10 % FCS and AAG. Then, 200 μl of the bacterial suspension were added. Two hours after infection, 30 $\mu\text{g/ml}$ gentamicin was added for the next 2 h. The cells were finally washed three times, before they were used as stimulator cells.

4.4 Responder T cells and APC

Peripheral blood was obtained from hemochromatosis patients as standard buffy coat preparations. PBMC were isolated by centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway). The interphase containing the PBMC was harvested and washed three times in cold PBS with 2 mM EDTA to eliminate the remaining platelets. PBMC were depleted of T cells by rosetting with 2-aminoethylisothiuronium (Sigma, St Louis, MO)-treated SRBC. Rosetted T cells were treated with 160 mM NH_4Cl to lyse the SRBC, and washed. CD8^+ T lymphocytes were isolated from rosetted T cells by positive selection using an anti-CD8 mAb coupled to magnetic microbeads (Miltényi Biotech, Germany). Sorted cells were frozen. The day before the first stimulation, CD8^+ T cells were thawed and cultured overnight in IMDM with 5 U/ml of IL-2, AAG and 10 % human serum. The lymphocyte-depleted PBMC were left to adhere for 2 h. Nonadherent cells were discarded and adherent cells were cultured in the presence of 100 U/ml IL-4 and 100 ng/ml GM-CSF in complete medium. Cultures were fed on day 2 by adding fresh medium with IL-4 and GM-CSF. They were frozen on day 4 and thawed the day before each stimulation. EBV-B cells were obtained following the standard protocol.

4.5 Activation of anti-MAGE CTL precursors

Autologous DC from donor LB1137 were infected for 2 h with the adeno-MAGE-A4 construct at a multiplicity of infection (MOI) of 200, and washed. CD8^+ T lymphocytes (150 000) and 30 000 infected DC were cocultured in micro-

wells in 200 µl IMDM, AAG and 10 % human serum and supplemented with 1 000 U/ml IL-6 and 10 ng/ml IL-12. The CD8⁺ lymphocytes were restimulated weekly with autologous DC freshly infected with adeno-MAGE-A4 and grown from day 7 in medium with 10 U/ml IL-2 and 5 ng/ml IL-7.

4.6 Cytotoxicity assay

Autologous EBV-B target cells were infected for 2 h with vaccinia constructs (MOI 20) before labeling for 1 h with Na⁵¹CrO₄. They were added to the responder cells of each microculture at an E/T ratio of ~40:1. To test the microcultures, unlabeled K562 cells were added (5 × 10⁴ per V-bottomed microwell) to block natural killer activity. Chromium release was measured after 4 h of incubation at 37 °C.

4.7 CTL clone 13: limiting dilution and culture conditions

A microculture containing cells with anti-MAGE-A4 lytic activity was cloned by limiting dilution using autologous EBV-B cells infected with a recombinant Yersinia-MAGE-A4 construct as stimulators, and allogeneic irradiated EBV-B cells (LG2-EBV) as feeder cells, in medium with 50 U/ml IL-2. CTL clone 13 was maintained in culture by weekly restimulation in 24-well plates with either EBV-B cells infected with Yersinia-MAGE-A4 (10⁵) or 0.5 µg/ml PHA in complete IMDM with 50 U/ml IL-2 and 10⁶ LG2-EBV feeder cells, as well as with 15 µg/ml gentamicin when Yersinia-infected cells were used as stimulator cells.

4.8 Construction of plasmids containing MAGE-A4 minigenes

To construct truncated MAGE-A4 cDNA, PCR reactions were performed using a MAGE-A4 cDNA as the template, an upstream primer consisting of the first nucleotides of the open reading frame of MAGE-A4 (position –7 to +20 in the MAGE-A4 open reading frame, position 0 corresponding to the A of the start codon) and eight downstream primers, separated from each other by approximately 100–120 bp in the open reading frame of MAGE-A4. The PCR was performed for 30 cycles (1 min at 94 °C, 2 min at 63 °C and 3 min at 72 °C). PCR products were ligated into the pcDNA3.1/V5/His-TOPO vector (Topo TA cloning kit, Invitrogen).

4.9 Transfection of COS-7 and HeLa cells and TNF assay

Transfection experiments were performed using DMRIE-C for COS-7 cells and LipofectAMINE for HeLa cells (both from GIBCO, Merelbeke, Belgium). Briefly, 15 000 COS-7 cells or 20 000 HeLa cells were treated in one microwell with 50 ng

plasmid pcDNA1/Amp containing the HLA-A2 genomic DNA, 50 ng plasmid containing the MAGE-A4 cDNA (truncated or not) and 1 µl of DMRIE-C or LipofectAMINE. These transfectants were tested for antigen expression in a TNF assay after 24 h, as previously described [44]. Briefly, 2 000 CTL were added to the microwells containing the cells, in a total volume of 100 µl IMDM complete medium with 25 U/ml IL-2. After 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on cells of WEHI-164 clone 13 in a MTT colorimetric assay [44–46].

4.10 IFN-γ assay

CTL (2500) were added to 20 000 DC in microplates containing 100 µl IMDM, AAG, 10 % human serum, and 25 U/ml IL-2. After 24 h, the supernatant was collected and its IFN-γ content was determined by ELISA developed in our laboratory using reagents from Biosource (Fleurus, Belgium).

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A *MAGE-A4* peptide presented by HLA-B37 is recognized on human tumors by cytolytic T lymphocytes

Key words:

antigenic peptide; CTL; HLA-B37; *MAGE-A4*; tumor

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Abbreviations:

CTL: cytolytic T lymphocyte; EBV-B cells: Epstein-Barr virus-transformed B cells.

Abstract: 'Cancer-germline' genes such as those of the *MAGE* family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They encode shared tumor-specific antigens, which have been used in therapeutic vaccination trials of cancer patients. *MAGE-A4* is expressed in more than 50% of carcinomas of esophagus, head and neck, lung, and bladder. We report here the identification of a new *MAGE-A4* encoded peptide, which is recognized by a cytolytic T lymphocyte (CTL) clone on HLA-B*3701. The sequence of the peptide is SESLKMIF. It corresponds to the *MAGE-A4*₁₅₆₋₁₆₃ protein sequence. When tumor cells expressing *MAGE-A4* were transfected with HLA-B*3701, they were recognized by the CTL clone, demonstrating that the peptide ought to be processed in tumor cells and could therefore serve as a target for therapeutic antitumoral vaccination.

MAGE-A4 belongs to the *MAGE* gene family which comprises 24 related functional genes divided into three clusters, named *MAGE-A*, *B*, and *C* (1–4). These genes are expressed in many human tumors of different histological types, but are silent in normal cells with the exception of testis. Some, including *MAGE-A4*, are also expressed in placenta (1). Male germline cells and placenta do not express MHC class I molecules and are therefore incapable of presenting antigens to cytolytic T lymphocyte (CTL) (5,6). *MAGE*-encoded antigens are thus tumor-specific, and are of particular interest for cancer immunotherapy because they are shared by many tumors. Clinical trials involving defined tumor-specific shared antigens have been and are being performed in melanoma patients, and tumor regressions have been observed in a minority of patients (7–10).

There are two known alleles of gene *MAGE-A4*, *MAGE-A4a* and *MAGE-A4b*. They code for proteins that differ by a single amino acid (11). The gene contains eight alternative first exons that are

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spliced to unique second and third exons. The entire open reading frame is located in the third exon. The role of the different first exons, each with their own promoter, is unknown. We have used in this work the coding sequence of *MAGE-A4a*, referred to hereafter as *MAGE-A4*. *MAGE-A4* is expressed in a significant proportion of tumors (Table 1).

We reported previously the identification of peptide GVDYD-REHTV (*MAGE-A4*₂₃₀₋₂₃₉), which is recognized by cytolytic T lymphocytes (CTL) on HLA-A2 (12). Only one antigenic peptide has been hitherto identified in the *MAGE-A4* protein. The identification of a large number of antigenic peptides presented by HLA class I and class II is likely to be important for the future of clinical trials with defined antigenic peptides. A large set of peptide/HLA combinations will alleviate HLA restriction and widen the set of eligible patients. It will also facilitate the design of concurrent immunizations against several antigens. Such immunizations could increase the primary antitumor efficacy of the vaccine and also decrease the risk of tumor escape by loss of antigen expression.

To isolate the first *MAGE-A4*-specific CTL, we had stimulated CD8⁺ T cells from non-cancerous blood donors with autologous dendritic cells infected with an adenovirus carrying the coding sequence of *MAGE-A4*. Here, we have used a slightly different strategy: the dendritic stimulator cells were infected with an avian poxvirus, ALVAC, carrying the coding sequence of *MAGE-A4*. We describe here the identification of a new *MAGE-A4* antigen.

Materials and methods

Cell lines, media, and reagents

The Epstein-Barr Virus-transformed B (EBV-B) cell lines and the melanoma cell lines were cultured in IMDM supplemented with 10% FCS. 293-EBNA cells were maintained in DMEM supplemented with 10% FCS. All culture media were purchased from GibcoBRL (Paisley, UK) and supplemented with 0.24 mM of L-asparagine, 0.55 mM of L-arginine, 1.5 mM of L-glutamine (AAG), 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Cell line MZ2-MEL 2.2-*MAGE-A4* was obtained by cotransfecting into MZ2-MEL 2.2 a pcDNA1/Amp plasmid (Invitrogen) that contains the coding sequence of *MAGE-A4* together with vector pSvtkneoβ, which contains the coding sequence conferring resistance to geneticin (13). Human recombinant IL-2 was purchased from Eurocetus (Amsterdam, the Netherlands). IL-7 was purchased from Genzyme (Cambridge, MA), GM-CSF (Leucomax) from Schering-Plough (Brinny, Ireland) and IFN-γ from Peprotech (Rocky Hill, USA). Human recombinant IL-4, IL-6, and IL-12 were produced in our laboratory.

One U/ml of IL-6 is the concentration needed to obtain half-maximal proliferation of mouse 7TD1 cells (14). Geneticin was purchased from GibcoBRL.

Recombinant viruses and infection of cell lines

The recombinant canarypox ALVAC-*MAGE-A4*, the vaccinia-*MAGE-A4*, and the parental vaccinia viruses were provided by Aventis Pasteur (Lyon, France). Retroviral vector *MAGE-A4*-CSM encodes the full length *MAGE-A4* protein and the truncated form of the human low affinity nerve growth factor receptor (LNGFR). It was produced as previously reported (15). Epstein-Barr Virus-B cells were transduced by coculture with irradiated packaging cell lines producing the *MAGE-A4*-CSM vector in the presence of polybrene (8 µg/ml). After 72 h, lymphocytes were harvested and seeded in fresh medium. The percentage of infected cells was evaluated

Expression of gene *MAGE-A4* by tumoral tissues*

Histological type	Percentage	n†
Bladder carcinoma		
superficial (<T2)	23	(70)
infiltrating (= T2)	45	(53)
Breast carcinoma	6	(135)
Colorectal carcinoma	11	(46)
Esophageal squamous-cell carcinoma	74	(19)
Head & neck squamous-cell carcinoma	53	(85)
Hepatocellular carcinoma	16	(50)
Leukemia	1	(112)
Lung carcinoma		
squamous-cell carcinoma	59	(93)
adenocarcinoma	35	(43)
Melanoma		
primary lesions	18	(83)
metastases	28	(243)
Myeloma		
stages I-II	0	(11)
stage III	22	(27)
Pediatric rhabdomyosarcoma	22	(31)
Prostate carcinoma	0	(22)
Renal cell carcinoma	2	(44)
Sarcoma	33	(15)

*Expression was measured by RT-PCR on total RNA of surgical samples using a primer specific for *MAGE-A4*. All results were obtained by Francis Brasseur at the Brussels Branch of the Ludwig Institute for Cancer Research(25), except for hepatocellular carcinoma(26) and pediatric rhabdomyosarcoma(27).

†Number of tumors tested.

Table 1

48h later by flow cytometry for LNGFr expression with the mAb 20.4 (ATCC, Rockville, MD). The LNGFr-positive cells were purified by magnetic cell sorting using rat antimouse IgG1-coated beads (Dynabeads M-450, DYNAL A.S. N012 Oslo, Norway). Using the same protocol, melanoma cell lines AVL3-MEL and LB1751-MEL were transduced with retroviral vector B37-CSM, which codes for the HLA-B*3701 molecule and the truncated form of LNGFr.

Dendritic cells and CD8⁺ responder T cells

Peripheral blood was obtained from hemochromatosis patient LB2257 as standard buffy coat preparations, which were laid down on a 15-ml Lymphoprep layer (Axis-Shield PoCAS, Oslo, Norway) in 50-ml tubes. To minimize contamination of the blood cells by platelets, the tubes were first centrifuged at 200g for 20 min at room temperature. After removal of the top 20–25 ml, containing most of the platelets, the tubes were centrifuged at 300g for 20 min at room temperature. The interphase containing the PBMCs was harvested and washed three times (or more) in cold phosphate buffer solution with 2 mM of EDTA in order to eliminate the remaining platelets. To generate autologous dendritic cells, the PBMCs were depleted from the T lymphocytes by rosetting with sheep erythrocytes (Bio Mérieux, Marcy-l'Etoile, France) treated with 2-aminoethylisothiuronium (Sigma-Aldrich, Steinheim, Germany). Rosetted T cells were treated with NH₄Cl (160 mM) to lyse the sheep erythrocytes and washed. CD8⁺ T lymphocytes were isolated from the rosetted T cells by positive selection using an anti-CD8 mAb coupled to magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). They were then sorted through a magnet and subsequently frozen. The day before the coculture with the dendritic cells, the CD8⁺ T cells were thawed and grown overnight in IMDM supplemented with 10% human serum, AAG, and antibiotics (hereafter referred to as complete IMDM) in the presence of 5 U/ml of IL-2. The lymphocyte-depleted PBMCs were left to adhere for 1 h at 37°C in culture flasks (FALCON, Becton Dickinson) at a density of 10⁶ cells per cm² in RPMI 1640 supplemented with Hepes (2.38 g/l), AAG, antibiotics, and 10% FCS (hereafter referred to as complete RPMI medium). Nonadherent cells were discarded and the adherent cells were cultured in the presence of IL-4 (200 U/ml) and GM-CSF (70 ng/ml) in complete RPMI medium. Cultures were fed on days 2 and 4 by removing one-third of the volume and adding fresh medium with IL-4 (200 U/ml) and GM-CSF (70 ng/ml). They were frozen on day 6.

Mixed lymphocyte-dendritic cell culture

Dendritic cells (4×10^6) were infected with ALVAC-*MAGE-A4* at a multiplicity of infection (MOI) of 30 in 200 µl of complete RPMI

medium at 37°C under 5% CO₂. The infected dendritic cells were washed after 2 h. Autologous responder CD8⁺ T lymphocytes (1.5×10^5) were mixed with infected dendritic cells (3×10^4) in U-bottomed microwells in 200 µl of complete IMDM in the presence of IL-6 (1000 U/ml) and IL-12 (10 ng/ml). On days 6 and 13, autologous dendritic cells were thawed and infected the day after with ALVAC-*MAGE-A4*. The infected cells were used to restimulate the responder lymphocytes in medium supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml). The responder lymphocytes were assessed on day 21 for their capacity to lyse autologous EBV-B cells infected with vaccinia-*MAGE-A4*.

Cytotoxicity assay

The cytotoxicity of an aliquot of each microculture was tested on autologous EBV-B cells infected with either vaccinia-*MAGE-A4* or the parental vaccinia virus. Infection was performed on 2×10^6 target cells for 2 h at an MOI of 20 in 150 µl of complete RPMI medium. Infected cells were washed, labeled with 100 µCi of Na(⁶¹Cr)O₄, and were added to the responder cells at an effector/target (E:T) ratio of 40:1. Unlabeled K562 cells were also added (5×10^4 per V-bottom microwell) to block natural killer activity. Individual microcultures were tested on each target in duplicate. Chromium release was measured after incubation at 37°C for 4 h. The melanoma cell lines were labeled with Na(⁶¹Cr)O₄ as described earlier, and pulsed, if indicated, for 15 min with 1 µg/ml of peptide.

Isolation of CD8⁺ T-cell clone specific for *MAGE-A4*

T cells from the microculture with anti-*MAGE-A4* reactivity were cloned in U-bottomed microplates by limiting dilution in complete IMDM supplemented with IL-2 (50 U/ml) and 15 µg/ml of gentamicin using, as stimulators, irradiated (100 Gy) $5\text{--}15 \times 10^3$ autologous EBV-B cells transduced with a retrovirus coding for *MAGE-A4*. Allogeneic EBV-B cells ($5\text{--}15 \times 10^3$ irradiated LG2-EBV-B cells per well) were used as feeder cells. Established CTL clones were grown in complete IMDM supplemented with IL-2 (50 U/ml) and 3×10^5 CTL were passaged weekly with irradiated feeder cells (1.5×10^6 LG2 EBV-B cells per well in a 24-well plate) and irradiated autologous EBV-B cells were transduced with retrovirus coding for *MAGE-A4* (10^5 cells per well).

Transfection of 293-EBNA cells and TNF assay

293-EBNA cells (2×10^4) were distributed in flat-bottom microwells and cotransfected with pcDNAI/Amp containing the *MAGE-A4* cDNA (50 ng) and 50 ng of pcDNAI/Amp containing the coding se-

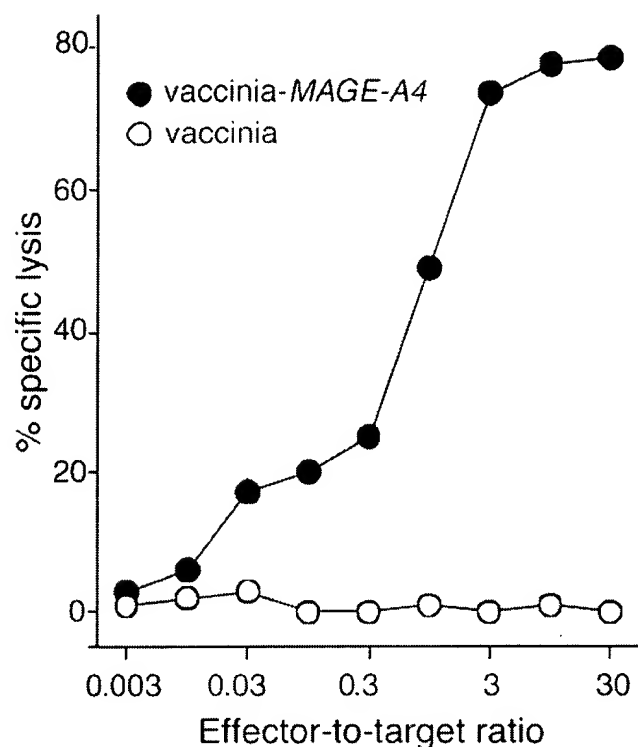


Fig. 1. Lysis by cytolytic T lymphocyte F2 of autologous EBV-B cells infected with vaccinia-*MAGE-A4*. Epstein-Barr Virus-B cells from donor LB2257 were infected for 2 h with the vaccinia vectors at a multiplicity of infection of 20, ^{51}Cr -labeled for 1 h, and incubated with autologous CTL F2 at the indicated effector-to-target ratios. Chromium release was measured after 4 h.

quences of each of the five putative HLA alleles using 1 μl of Lipofectamine (GibcoBRL). Transfected cells were incubated for 24 h at 37°C and 8% CO_2 . The transfectants were then tested for their ability to stimulate the production of TNF by the CTL clone. Briefly, 5000 CTL were added to the microwells containing the transfectants, in a total volume of 100 μl of complete IMDM supplemented with 25 U/ml of IL-2. After 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on WEHI-164 clone 13 cells in a MTT colorimetric assay (13,16,17).

Peptide recognition assay

Peptides were synthesized on solid phase using Fmoc for transient NH₂-terminal protection and were characterized using mass spectrometry. All peptides were >90% pure, as indicated by analytic HPLC. Lyophilized peptides were dissolved at 2 mg/ml in 10 mM of acetic acid and 10% DMSO, and were stored at -20°C. The first screening was performed with autologous EBV-B cells incubated

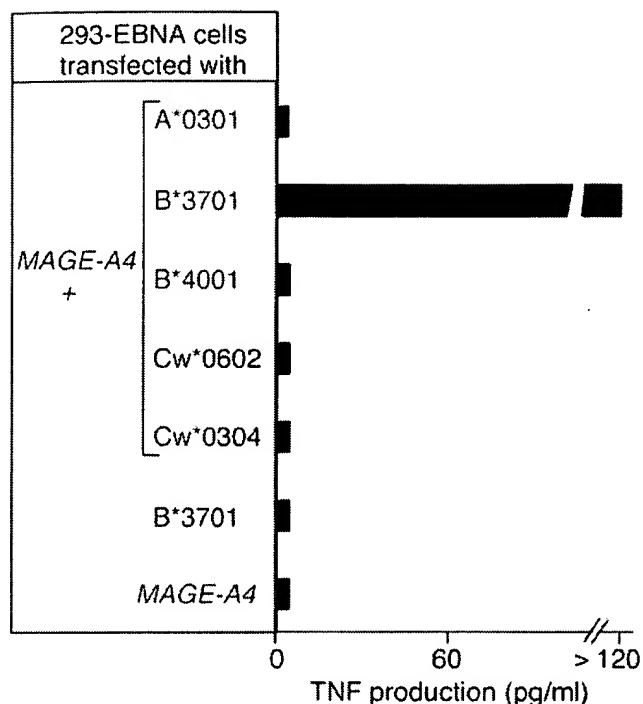


Fig. 2. The *MAGE-A4* antigenic peptide is presented by HLA-B37 molecules. 293-EBNA cells were transiently transfected with a *MAGE-A4* cDNA and each of the cDNAs coding for the putative HLA-presenting molecules. The cDNAs were inserted into expression vector pcDNA1/Amp. Transfections were performed in microwells with 20,000 293-EBNA cells, 50 ng of each cDNA and 1 μl of Lipofectamine. One day after transfection, 5000 cytolytic T lymphocyte F2 were added to the transfected cells. TNF production was measured after overnight coculture by testing the toxicity of the supernatants for TNF-sensitive WEHI 164 clone 13 cells.

with 16 amino-acid long peptides at a concentration of 1 $\mu\text{g}/\text{ml}$. Peptide-pulsed targets were tested for recognition by CTL at an E:T ratio of 5:1.

Results and discussion

Isolation of an anti-*MAGE-A4* CTL clone

Monocyte-derived dendritic cells of blood donor LB2257 were infected with an avian poxvirus, ALVAC, carrying the *MAGE-A4* coding sequence (ALVAC-*MAGE-A4*). These antigen-presenting cells were distributed in 96 microwells and were used to stimulate autologous CD8⁺ T lymphocytes in the presence of IL-6 and IL-12. After two weekly restimulations with dendritic cells infected with ALVAC-

MAGE-A4 in the presence of IL-2 and IL-7, the responder cells were tested on day 21 for their specific lytic activity on the autologous EBV-B cells infected with a vaccinia poxvirus encoding *MAGE-A4* (vaccinia-*MAGE-A4*). The reason for using vaccinia instead of ALVAC was that EBV-B cells infected with ALVAC are poor targets for cytotoxicity assays (data not shown). EBV-B cells infected with the parental vaccinia were used as control targets because CTL can recognize antigens shared by ALVAC and vaccinia poxvirus (18).

One microculture with anti-*MAGE-A4* lytic activity was obtained. The responder lymphocytes were cloned by limiting dilution using, as stimulator cells, autologous EBV-B cells transduced with a retrovirus carrying the coding sequence of *MAGE-A4*. This new stimulation combination was used to stimulate the anti-*MAGE-A4* CTL, while avoiding restimulating CTL directed against poxvirus antigens. We obtained stable CTL clone LB2257 661/F2 (hereafter re-

ferred to as CTL F2), which lysed the EBV-B cells infected with vaccinia-*MAGE-A4* but not the EBV-B cells infected with the parental vaccinia (Fig.1).

Identification of the HLA presenting molecule

Blood donor LB2257 was typed HLA-A*0301, B*3701, B*4001, Cw*0302 and Cw*0602. To identify the HLA molecule that presents the *MAGE-A4* peptide recognized by CTL F2, 293-EBNA cells, which derived from a human embryonic kidney cell line, were transiently transfected with a *MAGE-A4* cDNA together with each of the cDNAs encoding the putative HLA presenting molecules. Only those cells that were transfected with *MAGE-A4* and HLA-B*3701 stimulated CTL F2 to produce TNF (Fig.2).

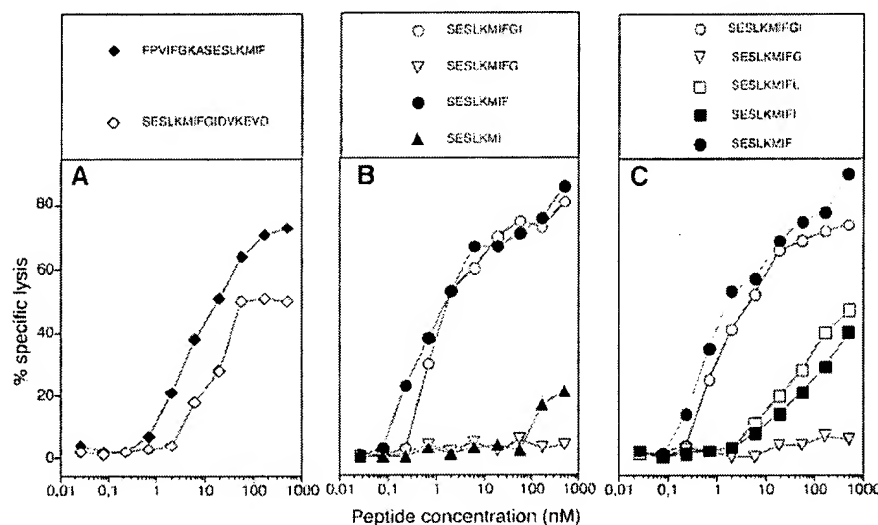


Fig. 3. Peptide titration. (A) Identification of two 16-mer peptides recognized by cytolytic T lymphocyte (CTL) F2. (B) Identification of the shortest peptide recognized by CTL F2. (C) Test of peptides modified at the C-terminus. Autologous EBV-B cells were ^{51}Cr -labeled and incubated for 15 min with threefold dilutions of synthetic peptides. Autologous CTL F2 was subsequently added at an effector-to-target ratio of 5:1. Chromium release was measured after 4 h. The concentrations indicated in the figure are the concentrations during 4-h incubation. Experiments described in (B) and (C) were performed with HPLC-purified peptides (99% pure).

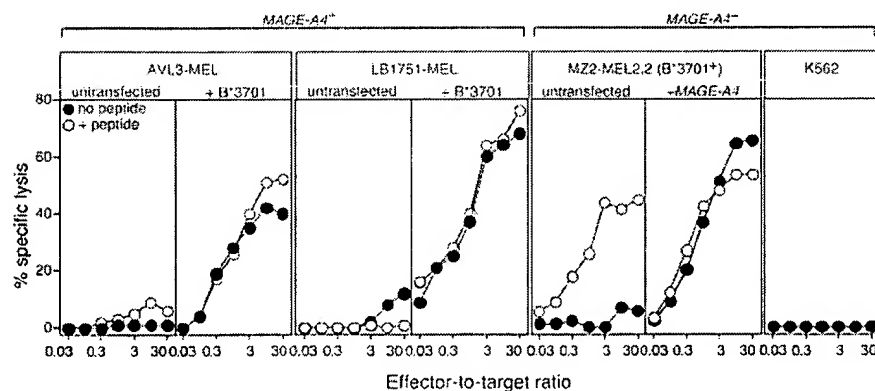


Fig. 4. Lysis by cytolytic T lymphocyte F2 of B*3701 tumor cell lines expressing *MAGE-A4*. Target cells were ^{51}Cr -labeled and, if indicated, pulsed for 15 min with 1 $\mu\text{g}/\text{ml}$ of peptide SESLKMIF. They were then incubated for 4 h with CTL F2 at the indicated effector-to-target ratios. Chromium release was measured after 4 h.

Identification of the antigenic peptide

To determine the peptide recognized by CTL F2, we screened a set of peptides covering the entire *MAGE-A4* protein sequence. These 16 amino-acid long peptides were overlapped by 12 residues. Autologous EBV-B cells were incubated with 1 µg/ml of each of these peptides and were tested for lysis by CTL F2. Peptide FPVIFGKA-SESLKMIF (*MAGE-A4*₁₄₈₋₁₆₃) and SESLKMIFGIDVKEVD (*MAGE-A4*₁₅₆₋₁₇₁) scored positive (Fig. 3A). The consensus anchor residues for HLA-B37 are D/E in position 2, F/M/L in position 8, and I/L in the C-terminal position (19,20). One peptide, SESLKMIFGI, contains these three anchor residues but its sequence was only included in one of the two long peptides that scored positive. We therefore tested a number of peptides of different lengths. Peptide SESLKMIFGI and peptide SESLKMIF scored positive and half maximal lysis of autologous EBV-B target cells was obtained at a peptide concentration of 1 nM (Fig. 3B). This is within the range of the previously identified MAGE antigenic peptides, for which values ranging from 0.05 to 100 nM were observed (18,21–24). These two peptides are encoded by both *MAGE-A4a* and *MAGE-A4b*.

Interestingly, nonapeptide SESLKMIFG was not recognized by CTL F2 (Fig. 3B). The C-terminal residue G was replaced by a consensus anchor residue for B37, either a I or a L (19). Targets pulsed with a very high and most probably not physiological concentration of these two modified peptides were recognized by the CTL (Fig. 3C). The results suggest that the C-terminal residue is very important

for the binding to B37 and that the presence of a G decreases the affinity of the peptide for B37.

Lysis of tumor cell lines

As we used dendritic cells expressing *MAGE-A4* to activate CTL F2, it was important to verify that tumor cells also process the *MAGE-A4* antigen. Because we had no tumor cell lines expressing both *MAGE-A4* and B*3701, we transduced *MAGE-A4*-expressing melanoma cell lines with a retrovirus carrying the B*3701 coding sequence. The B*3701-transduced cell lines were both lysed by CTL F2 (Fig. 4). Melanoma cell line MZ2-MEL.2.2, which expressed B*3701 but not *MAGE-A4*, was transduced with a retrovirus carrying the *MAGE-A4* coding sequence. It was efficiently lysed after transduction (Fig. 4). Control K562 cells were not lysed.

The new *MAGE-A4* antigenic peptide described here is presented by B*3701 molecules. The HLA allelic group B37 comprises two alleles. B*3701 corresponds to the B37 serological specificity, but B*3702 is not recognized by serum antibodies and it is therefore considered as 'blank'. B37 molecules are rarely expressed in the different major ethnic groups: Black (2%), Caucasoid (3%), Oriental (2%), Amerindian (4%). Identification of additional *MAGE-A4* antigenic peptides will be important because a number of tumors express *MAGE-A4* without expressing *MAGE-A1* and *MAGE-A3*. It is the case for carcinomas of the lung (15%), the head and neck (14%), the esophagus (11%), and the bladder (10%).

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